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(54) Title: CHIMERIC FLAVIVIRUS VECTORS

(57) Abstract: The invention provides chimeric flavivirus vectors including foreign peptides inserted into the envelope proteins of the vectors and methods of using these vectors.

CHIMERIC FLAVIVIRUS VECTORS

Field of the Invention

This invention relates to chimeric flavivirus vectors and methods employing these vectors.

Background of the Invention

Flaviviruses are small, enveloped positive-strand RNA viruses. Flavivirus proteins are produced by translation of a single, long open reading frame to generate a polyprotein, which is followed by a complex series of post-translational proteolytic cleavages of the polyprotein by a combination of host and viral proteases to generate mature viral proteins (Amberg et al., J. Virol. 73:8083-8094, 1999; Rice, "Flaviviridae," In *Virology*, Fields (ed.), Raven-Lippincott, New York, 1995, Volume I, p. 937). The virus structural proteins are arranged in the polyprotein in the order C-prM-E, where "C" is capsid, "prM" is a precursor of the viral envelope-bound M protein, and "E" is the envelope protein. These proteins are present in the N-terminal region of the polyprotein, while the non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are located in the C-terminal region of the polyprotein.

A chimeric flavivirus that includes the C and non-structural proteins of the Yellow fever virus vaccine strain (YF 17D) and the prM and E proteins of a strain of attenuated Japanese encephalitis virus (SA 14-14-2) has been made. This chimera, designated ChimeriVax™-JE, has been shown to induce the production of neutralizing antibodies against JE in immunized rhesus monkeys, as well as to protect these monkeys from clinical encephalitis after JE challenge, as compared with unimmunized controls. ChimeriVax™-JE was shown to meet preclinical safety requirements for a human vaccine (Monath et al., J. Virol. 74(4):1742-1751, 2000).

A similar chimera was made that includes the C and non-structural proteins of YF 17D and the prM and E proteins of a Dengue-2 strain. This chimera, designated ChimeriVax-D2, was shown to induce neutralizing antibodies against Dengue-2 virus in rhesus monkeys, as well as to protect these monkeys from viremia after Dengue-2

challenge, as compared with unimmunized controls. ChimeriVax-D2 also was shown to be safe and genetically stable (Guirakhoo et al., J. Virol. 74(12):5477-5485, 2000).

Summary of the Invention

The invention provides methods for identifying sites in the envelope proteins of chimeric flaviviruses or genetically attenuated flaviviruses that are permissive for insertion of foreign peptides. These methods include the steps of: (i) introducing a nucleic acid molecule encoding a foreign peptide into a gene encoding a flavivirus envelope protein; (ii) generating a flavivirus vector including an envelope protein encoded by the gene, wherein the envelope protein contains the foreign peptide; and (iii) determining whether the flavivirus vector generated in step (ii) is permissive for the insertion.

The flavivirus vectors can be chimeric flavivirus vectors that include, for example, the C and non-structural proteins of a first flavivirus and the prM and E proteins of a second flavivirus. The first and second flaviviruses can be selected from the group consisting of Japanese encephalitis, Dengue (serotype 1, 2, 3, or 4), Yellow fever (e.g., YF 17D), Murray Valley encephalitis, St. Louis encephalitis, West Nile, Kunjin, Rocio encephalitis, Ilheus, ticke-borne encephalitis, Central European encephalitis, Siberian encephalitis, Russian Spring-Summer encephalitis, Kyasanur Forest Disease, Omsk Hemorrhagic fever, Louping ill, Powassan, Negishi, Absettarov, Hansalova, Apoi, and Hypr viruses.

The foreign peptides inserted into the vectors of the invention can include epitopes derived from, for example, antigens of viral, bacterial, or parasitic pathogens, or can include epitopes derived from tumor-associated antigens. Examples of these peptides and others are provided below.

The nucleic acid molecules can be introduced into the envelope genes of the flaviviruses, according to the methods of the invention, for example, randomly by transposon mutagenesis. Also, determination of whether the flavivirus vectors generated in step (ii) of the methods of the invention are permissive for the insertion can be carried out, for example, by analysis of (a) the infectivity of the flavivirus vectors, (b) the stability of the sequence of the foreign protein upon multiple passages of the vectors, (c) the growth properties of the flavivirus vectors, and/or (d) whether the flavivirus vectors can be neutralized with antibodies against the envelope protein of the first flavivirus.

The methods of the invention can further include comparing the analysis of the flavivirus vectors with a similar analysis of the flavivirus from which it was derived.

The invention also includes flavivirus vectors that include envelope proteins that contain foreign peptides. The flavivirus vectors can be chimeric flaviviruses including the prM and E proteins of a first flavivirus and the C and non-structural proteins of a second flavivirus. The first and second flaviviruses can be selected from the group consisting of Japanese encephalitis, Dengue (serotype 1, 2, 3, or 4), Yellow fever (e.g., YF 17D), Murray Valley encephalitis, St. Louis encephalitis, West Nile, Kunjin, Rocio encephalitis, Ilheus, ticke-borne encephalitis, Central European encephalitis, Siberian encephalitis, Russian Spring-Summer encephalitis, Kyasanur Forest Disease, Omsk Hemorrhagic fever, Louping ill, Powassan, Negishi, Absettarov, Hansalova, Apoi, and Hypr viruses. The flavivirus vectors can, alternatively, be a genetically attenuated flavivirus, such as Yellow Fever YF 17D.

The foreign peptides inserted into the vectors can include epitopes derived from antigens of viral, bacterial, or parasitic pathogens. Alternatively, the foreign peptides can include epitopes derived from tumor-associated antigens.

Also included in the invention are pharmaceutical compositions that include the flavivirus vectors described above and pharmaceutically acceptable carriers or diluents, as well as methods of delivering peptides to patients by administering to the patients such compositions. These methods can be carried out, for example, when the peptides are antigens, to induce an immune response to pathogens or tumors from which the antigens are derived. The invention also includes use of these compositions for the delivery of peptides, as well as their use in the preparation of medicaments for delivery of peptides.

The invention also includes nucleic acid molecules that include the genomes of the flaviviruses described above or the complements thereof.

The invention provides several advantages. For example, chimeric flavivirus vectors that can be used in the invention are sufficiently attenuated so as to be safe, and yet are able to induce protective immunity to the flaviviruses from which the envelope proteins in the chimeras are derived and, in particular, the epitopes inserted into the chimeras. Additional safety comes from the fact that the vectors used in the invention are chimeric, thus eliminating the possibility of reversion to wild type. An additional advantage of the vectors used in the invention is that flaviviruses replicate in the

cytoplasm of cells, so that the virus replication strategy does not involve integration of the viral genome into the host cell, providing an important safety measure. In addition, as is discussed further below, a single vector of the invention can be used to deliver multiple epitopes from a single antigen, or epitopes derived from more than one antigen.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

Brief Description of the Drawings

Fig. 1 is a schematic representation of a transposon mutagenesis method carried out with the gene encoding the JE envelope.

Fig. 2 shows the results of PCR analysis of Tn5 insertion mutants of the gene encoding the JE envelope. Stable clones harboring a transposon in the JE envelope are shown in lanes 1, 2, 8, and 10; clones harboring a transposon within the pUC19 vector are shown in lanes 3 and 4; unstable clones harboring a transposon in the JE envelope are shown in lanes 5-7 and 9; and a 1 kilobase marker is shown in lane 11.

Fig. 3 shows the results of PCR mapping of select mutant plasmids. This analysis confirmed the random nature of the insertion into the JE envelope. Lanes 1-13 show the PCR products of 13 clones, while lane 14 contains a 1 kilobase marker.

Fig. 4 shows the amino acid sequences of five mutant clones that were selected for transfection of Vero cells. The JE envelope sequences are in boldface and the insert sequences following transposition are underlined. The remaining sequences, which are repeated sets of three amino acids each that are on the left side of the first dash and on the left side of the second dash in each line, are artifacts of the transposon mutagenesis.

Fig. 5 is a schematic representation of the envelope glycoprotein of JE, which shows the locations of inserts relative to defined conformational epitopes of the protein. The arrows pinpoint the approximate insertion sites for 5 independent JE envelope mutants that were selected for transfection of Vero cells. The numbers in brackets indicate the JE envelope amino acid that precedes the 19 amino acid insert.

Fig. 6 shows the results of RT-PCR analysis of cDNA synthesized from RNA extracted from Vero cells that have been transfected with RNA made from 5 unique clones (2 in duplicate). A 1.9 kilobase insert that includes the 57 basepair linker was amplified from each of the clones, and confirms the production of progeny virus. A 1

kilobase marker is fractionated in lane 1. Lanes 2-8 correspond to clone I-10-2, clone I-10-3, clone II-4-3, clone II-4-6, clone I-1, clone III-6, and clone IV-8-1, respectively.

Fig. 7 shows the results of PCR analysis of clone I-10 cDNA at P2 through P6 (lanes 2-6 and 8-12, respectively). The samples fractionated on the left panel were obtained using JE envelope specific primers, while the samples fractionated on the right panel were obtained using a transposon-specific primer. A 1 kilobase marker is fractionated in lane 1 and a 100 basepair marker is fractionated in lane 7.

Fig. 8 shows the nucleotide sequences of the RT-PCR products of infectious clone I-10 at P2 through P6, aligned with the plasmid I-10 sequence prior to transfection. The arrows indicate two cysteine residues in the insert that have the potential to form a disulfide bond, possibly stabilizing the peptide on the envelope protein and presenting it on the surface of the molecule.

Fig. 9 is a schematic representation of the 3-dimensional structure of the JE envelope glycoprotein, showing that the permissive site at amino acid position 287 is located between the central (I) and the dimerization (II) domains, and appears to be surface exposed.

Fig. 10 is a graph showing the growth properties of the I-10 infectious clone. The line with the triangles corresponds to the titer of the transposon mutant I-10, while the line with the squares corresponds to the titer of the YF/JE parent virus.

Fig. 11 is a graph showing the results of a neutralization assay of transposon mutant I-10. The line with the diamond corresponds to the titer of samples incubated with anti-JE serum, the squares correspond to samples incubated with normal serum, and the dashed line shows the average.

Fig. 12 is a graph showing the results of a neutralization assay of the YF/JE reference. The line with the diamond corresponds to the titer of samples incubated with anti-JE serum, the squares correspond to samples incubated with normal serum, and the dashed line shows the average.

Detailed Description

The invention provides methods of identifying sites in the envelope proteins of chimeric flaviviruses or genetically attenuated flaviviruses (e.g., YF 17D) into which foreign peptides can be introduced, chimeric flavivirus vectors having envelope proteins that include such peptides, and methods of delivering these peptides by administration of

the vectors in order to, for example, induce an immune response to a pathogen from which an introduced peptide is derived. Details of these vectors, peptides, and methods are provided below.

Chimeric Flavivirus Vectors

Chimeric viruses that can be used in the invention consist of a first flavivirus (i.e., a backbone flavivirus) in which a structural protein (or proteins) has been replaced with a corresponding structural protein (or proteins) of a second virus. For example, the chimeras can consist of a first flavivirus in which the prM and E proteins have been replaced with the prM and E proteins of a second virus.

The chimeric viruses that are used in the invention can be made from any combination of viruses. Examples of particular flaviviruses that can be used in the invention, as first or second viruses, include mosquito-borne flaviviruses, such as Japanese encephalitis, Dengue (serotypes 1-4), Yellow fever, Murray Valley encephalitis, St. Louis encephalitis, West Nile, Kunjin, Rocio encephalitis, and Ilheus viruses; tick-borne flaviviruses, such as Central European encephalitis, Siberian encephalitis, Russian Spring-Summer encephalitis, Kyasanur Forest Disease, Omsk Hemorrhagic fever, Louping ill, Powassan, Negishi, Absettarov, Hansalova, Apoi, and Hypr viruses; as well as viruses from the Hepacivirus genus (e.g., Hepatitis C virus).

Details of making chimeric viruses that can be used in the invention are provided, for example, in U.S. patent application serial numbers 09/007,664, 09/121,587, and 09/452,638; International applications PCT/US98/03894 and PCT/US00/32821; and Chambers et al., J. Virol. 73:3095-3101, 1999, each of which is incorporated by reference herein in its entirety.

A specific example of a type of chimeric virus that can be used in the invention is the yellow fever human vaccine strain, YF 17D, in which the prM and E proteins have been replaced with prM and E proteins of another flavivirus, such as Japanese encephalitis virus, West Nile virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, a Dengue virus, or any other flavivirus, such as one of those listed above. For example, the following chimeric flaviviruses, which were deposited with the American Type Culture Collection (ATCC) in Manassas, Virginia, U.S.A. under the terms of the Budapest Treaty and granted a deposit date of January 6, 1998, can be used in the invention: Chimeric Yellow Fever 17D/Japanese Encephalitis SA14-14-2 Virus

(YF/JE A1.3; ATCC accession number ATCC VR-2594) and Chimeric Yellow Fever 17D/Dengue Type 2 Virus (YF/DEN-2; ATCC accession number ATCC VR-2593).

Methods for Identifying Permissive Sites in Chimeric Flavivirus Envelope Proteins

Sites in chimeric flavivirus envelope proteins that are permissive to insertion of foreign sequences can be identified as follows. Nucleic acid sequences encoding a peptide are inserted into the envelope gene using standard methods of molecular biology. Preferably, such nucleic acid sequences are randomly inserted into the envelope gene, to facilitate the creation of a library of insertion mutants. However, a nucleic acid sequence can, alternatively, be inserted a specific point in an envelope gene and tested for efficacy. The latter approach may be desirable, for example, when a particular site has been identified as being permissive for insertion of a first foreign sequence and it is desired to confirm that it is also permissive for insertion of a second sequence that may, for example, differ in length or predicted secondary structure from the first foreign sequence.

Random insertion of nucleic acid sequences can be achieved, for example, by the use of a transposon mutagenesis approach. For example, a Tn5 transposition system can be used (Goryshin et al., J. Biol. Chem. 273:7367, 1998). As a specific example, the EZ::TN Insertion System, which is manufactured by Epicentre Technologies (Madison, WI, U.S.A.), can be used. Details of the use of this system in the invention are provided further below. In summary, a cloned flavivirus envelope gene is subjected to mutagenesis with transposons that include sequences that encode peptides. A library of mutants that include randomly integrated transposons in flavivirus envelope genes is generated and, if desired, the insertion sites are mapped and/or sequenced. Full length genomic RNA that includes mutant envelope genes is then generated and used to make mutant viruses, which are then characterized for permissiveness to insertion of the transposons. The viruses can be analyzed for permissiveness by, for example, determination of infectivity, genomic stability, growth properties, and neutralization. Details of the use of this transposon mutagenesis system are provided below, in the context of the chimeric flavivirus ChimeriVax™-JE (also see Fig. 1). However, the methods can be used with any of the chimeras described herein.

Foreign Peptides

The vectors of the invention can be used in the delivery of any peptide or protein of prophylactic or therapeutic value. For example, the vectors of the invention can be used in the induction of an immune response (prophylactic or therapeutic) to any protein-based antigen that is inserted into a chimeric flavivirus envelope protein. Preferably, such an antigen not derived from the second flavivirus of the chimera. All that is required is that a nucleic acid sequence encoding the antigen be inserted at a permissive site within the envelope gene of a chimeric flavivirus, as described herein. Standard methods of molecular biology can be used to insert the antigen-coding nucleic acid molecules into chimera envelope genes, at permissive sites, which are identified as is described elsewhere herein.

The vectors of the invention can each include a single epitope. Alternatively, multiple epitopes can be inserted into the vectors, either at a single site (i.e., as a polytope, in which the different epitopes can be separated by a flexible linker, such as a polyglycine stretch of amino acids), at different sites, or in any combination thereof. The different epitopes can be derived from a single species of pathogen, or can be derived from different species and/or different genuses.

Antigens that can be used in the invention can be derived from, for example, infectious agents such as viruses, bacteria, and parasites. For example, antigens from the pathogens listed in Table 2, below, can be used. Specific examples of such antigens include those listed in Table 3. In addition, specific examples of epitopes that can be inserted into the vectors of the invention are provided in Table 4. As is noted in Table 4, epitopes that are used in the vectors of the invention can be B cell epitopes (i.e., neutralizing epitopes) or T cell epitopes (i.e., T helper and cytotoxic T cell-specific epitopes).

The vectors of the invention can be used to deliver antigens in addition to pathogen-derived antigens. For example, the vectors of the invention can be used to deliver tumor-associated antigens for use in immunotherapeutic methods against cancer. Numerous tumor-associated antigens are known in the art and can be used in the invention. Examples of cancers (and corresponding tumor associated antigens) are as follows: melanoma (NY-ESO-1 protein (specifically CTL epitope located at amino acid positions 157-165), CAMEL, MART 1, gp100, tyrosine-related proteins TRP1 and 2, and MUC1)); adenocarcinoma (ErbB2 protein); colorectal cancer (17-1A, 791Tgp72,

and carcinoembryonic antigen); prostate cancer (PSA1 and PSA3). Heat shock protein (hsp110) can also be used as such an antigen.

In another embodiment of the invention, exogenous proteins that encode an epitope(s) of an allergy-inducing antigen to which an immune response is desired may be used. In addition, the vectors of the invention can include ligands that are used to target the vectors to deliver peptides, such as antigens, to particular cells (e.g., cells that include receptors for the ligands) in subjects to whom the vectors administered.

The size of the peptide or protein that is inserted into the vectors of the invention can range in length from, for example, from 5-500 amino acids in length, for example, from 10-100, 20-55, 25-45, or 35-40 amino acids in length. The feasibility of using any particular desired peptide can easily be determined using the methods described herein.

Use of Chimeric Flavivirus Vectors to Deliver Foreign Peptides

The vectors of the invention are administered in amounts and by using methods that can readily be determined by persons of ordinary skill in this art. The vectors can be administered and formulated, for example, in the same manner as the yellow fever 17D vaccine, e.g., as a clarified suspension of infected chicken embryo tissue, or a fluid harvested from cell cultures infected with the chimeric yellow fever virus. Thus, the vectors of the invention can be formulated as sterile aqueous solutions containing between 100 and 1,000,000 infectious units (e.g., plaque-forming units or tissue culture infectious doses) in a dose volume of 0.1 to 1.0 ml, to be administered by, for example, intramuscular, subcutaneous, or intradermal routes. In addition, because flaviviruses may be capable of infecting the human host *via* the mucosal routes, such as the oral route (Gresikova et al., "Tick-borne Encephalitis," In *The Arboviruses, Ecology and Epidemiology*, Monath (ed.), CRC Press, Boca Raton, Florida, 1988, Volume IV, 177-203), the vectors can be administered by a mucosal route.

When used in immunization methods, the vectors can be administered as a primary prophylactic agent in adults or children at risk of infection by a particular pathogen. The vectors can also be used as secondary agents for treating infected patients by stimulating an immune response against the pathogen from which the peptide antigen is derived.

For vaccine applications, adjuvants that are known to those skilled in the art can be used. Adjuvants that can be used to enhance the immunogenicity of the chimeric vectors include, for example, liposomal formulations, synthetic adjuvants, such as (e.g., QS21), muramyl dipeptide, monophosphoryl lipid A, or polyphosphazine. Although these adjuvants are typically used to enhance immune responses to inactivated vaccines, they can also be used with live vaccines. In the case of a chimeric vector delivered via a mucosal route, for example, orally, mucosal adjuvants such as the heat-labile toxin of *E. coli* (LT) or mutant derivations of LT can be used as adjuvants. In addition, genes encoding cytokines that have adjuvant activities can be inserted into the vectors. Thus, genes encoding cytokines, such as GM-CSF, IL-2, IL-12, IL-13, or IL-5, can be inserted together with foreign antigen genes to produce a vaccine that results in enhanced immune responses, or to modulate immunity directed more specifically towards cellular, humoral, or mucosal responses.

In addition to vaccine applications, as one skilled in the art can readily understand, the vectors of the invention can be used in gene therapy methods to introduce therapeutic gene products into a patient's cells and in cancer therapy. In these methods, genes encoding therapeutic gene products are inserted into permissive sites in the vectors.

Example

The following experimental example shows the identification of permissive sites in ChimeriVax™-JE. The methods described in this example can be used with other chimeric flaviviruses, such as those described above, as well.

The Yellow fever 17D (YF 17D) live attenuated vaccine strain has been used in humans for the past 60 years, has an excellent safety record, and provides long-lasting immunity after administration of a single dose. As is noted above, ChimeriVax™-JE is a live, attenuated recombinant vaccine strain in which the genes encoding the structural proteins (PrME) of YF 17D have been replaced with the corresponding genes from the genetically attenuated Japanese encephalitis (JE) virus SA14-14-2. Both capsid and all nonstructural (NS) genes responsible for intracellular replication of this chimera are derived from the YF 17D vaccine strain. As is noted above, an infectious molecular clone of ChimeriVax™-JE (YF/JE) has previously been described. In the experiments

described below, ChimeriVax™-JE was evaluated as to its suitability as a delivery vehicle for biologically relevant peptides.

The EZ::TN In-Frame Linker Insertion Kit© (Epicentre) is a fast and efficient method for randomly inserting 19 amino acid peptides in-frame into proteins encoded by cloned DNA for a variety of applications. Using this approach, we have chosen to identify sites within the envelope gene of ChimeriVax™-JE that are permissive to foreign DNA. As is discussed in further detail below, random mutagenesis in *E. coli* of the gene encoding the JE envelope protein with EZ::TN identified a bank of stable insertion mutants that carried the 57 basepairs fragment that encodes the 19 amino acid peptide. DNA sequence analysis, restriction mapping, and PCR studies confirmed both the exact location of the transposon and the random nature of insertion. Engineering the mutated JE envelope gene back into the ChimeriVax™-JE infectious clone has allowed us to study infection in cell culture and provide valuable information on the use of recombinant flaviviruses as delivery vehicles for foreign antigens. We identified a panel of mutant clones infectious for Vero cells and characterized their biological properties. Specifically, we compared the growth properties of stable infectious clones to the parental ChimeriVax™-JE chimera in cell culture, as well as their ability to be neutralized in a plaque reduction neutralization test (PRNT) with JE-specific polyclonal antisera. We identified sites within the JE envelope that are permissive to insertion of foreign DNA, and these sites can be exploited for delivery of biologically relevant epitopes. Further details are provided as follows.

Cloning of the gene encoding the JE envelope into pUC19

YF/JE viral RNA was extracted from infected Vero cells using Trizol reagent (Gibco BRL). Following cDNA synthesis with the FNOR antisense primer (see below), the gene encoding the JE SA14-14-2 envelope was amplified by XL-PCR with TN1.F/TN2.R primers (see below), and directionally cloned by conventional methods into pUC19 (New England Biologicals, NEB, U.S.A.) using KpnI and PstI recognition sequences incorporated at the 5' ends of each oligonucleotide, generating pJEe1. PCR was carried out using a GeneAmp PCR System 2400 (Perkin Elmer).

Transposon mutagenesis and mapping of insertion sites

Insertion mutagenesis was performed on pJEe1 using the EZ::TN™ In-Frame Linker Insertion Kit (EPICENTRE Technologies, U.S.A.), according to manufacturer's instructions. The EZ::TN <NotI/Kan-3> transposon contains a kanamycin resistance gene flanked by Not I restriction sites. As is discussed further below, removal of the kanamycin resistance gene following NotI digestion and re-ligation generates a 19 codon insertion that can be read in all three reading frames.

Insertion mutants were identified in *E. coli* by selection on LB agar plus kanamycin (50 μg/ml). PCR with TN1.F/TN2.R on select kanamycin-resistant clones following transposition revealed that 40% of clones stably maintained the transposon in the JE envelope (Fig. 2). Insertion sites were mapped by PCR (Pwo DNA Polymerase, Boehringer Mannheim/Roche) using Tn5-specific primers TN1.F and NotI/KAN-3 RP-2, which showed that the Tn5 transposon inserted randomly into the JE envelope (Fig. 3). Unique clones were selected for sequencing and generation of full length mutant genomic RNA.

Sequencing

The amino acid sequences of five mutant clones that were selected for transfection of Vero cells is shown in Fig. 4. The locations of the inserts relative to defined conformation epitopes on the envelope protein of JE virus is shown in Fig. 5. The insertion sites were determined by DNA sequencing, and inserts were located by comparison to a predicted 3D structure of the JE envelope (Kolaskar et al., Virology 261:31-42, 1999). Four of the five insertion sites appear to be surface exposed. Sequencing was performed using a CEQ™ 2000 DNA Analysis system (Beckman Coulter) and a CEQ 2000 Dye Terminator Cycle Sequencing Kit. Data were analyzed using SEQUENCHER™, Version 4.0.5. (Gene Codes Corporation).

Constructing infectious clones harboring Tn5 insertions

The antibiotic resistance marker was removed from stable *E. coli* clones, which were then re-ligated, leaving a 57 basepair in-frame insertion that included a 9-basepair target site sequence duplication that flanks the transposon. Sample clones (n=5) containing re-ligated JE envelope were then digested with NheI/NarI (NEB, U.S.A.), to

be compatible for cloning into a two plasmid system previously described for generating full length YF/JE genomes (Chambers et al., 1999, supra).

Transcription and transfection

Transcription of linearized, full length genomic DNA harboring foreign DNA in the gene encoding the JE envelope was performed from the SP6 promoter using the AmpliScribe™ SP6 High Yield Transcription Kit (EPICENTRE Technologies). Six well plates seeded with Vero cells were transfected with *in vitro* transcribed genomic RNA in the presence of LIPOFECTIN Reagent (Life Technologies) and maintained in MEM (Life Technologies) supplemented with 5% FBS (Hyclone), NEAA (Life Technologies), and 1% Penicillin-Streptomycin (Sigma Chemicals). Cell supernatants (500 mL) were passaged to fresh cells every 6 days through P6, and the monolayer was monitored for cytopathic effects (CPE). Viral RNA was extracted from the cell monolayer and supernatant at each passage.

As is shown in Fig. 6, all mutant clones were infectious for Vero cells at P2. In particular, RT-PCR using TN1.F/TN2.R on cDNA synthesized from RNA extracts of cell monolayers amplified a 1.9 kilobase insert that harbors the 57 basepair linker, and confirms the production of progeny virus. PCR analysis of clone I-10 from P2 through P6 shows that this clone is stable through P6, and that its insertion site, at amino acid 287, is permissive for the insertion of foreign DNA. PCR was carried out with JE envelope gene-specific primers (TN1.F/TN2.R) and a transposon-specific primer (TN1.F/TMOS.R) (Fig. 7).

The DNA sequence of RT PCR products from P2 through P6 was determined and, as is shown in Fig. 8, the sequences at each passage were identical to the sequence of the original clone, I-10. Interestingly, the I-10 transposon insert contains two cysteine residues that have the potential to form a disulfide bond, which possibly stabilizes the foreign peptide on the envelope protein and presents it on the surface of the molecule. Fig. 9 depicts a 3-D structure of the JE virus envelope glycoprotein, and shows that position 287 is located between the central (I) domain and the dimerization (II) domain and appears to be surface exposed.

The biological properties of transposon mutant I-10 were determined and compared with those of ChimeriVax™-JE. First, the growth properties of clone I-10 were determined. As is shown in Fig. 10, the mutant infectious clone I-10 shows similar

growth kinetics as the ChimeriVax™-JE parent, but grows to a slightly lower titer, reaching 8e5 PFU/ml, as compared to 1e7 PFU/ml for YF/JE. Also, the mutant infectious clone I-10 was found to induce a cytopathic effect in Vero cells, analogous to its YF/JE parent.

Plaque reduction neutralization (PRNT) assays were then carried out. A preparation of anti-JE polyclonal antisera was shown to neutralize clone I-10 and ChimeriVax™-JE to the same degree (1:64,000), confirming the feasibility of inserting foreign DNA into the envelope of the JE chimera without unduly affecting the structural integrity of the viral envelope (Figs. 11 and 12). These experiments show that amino acid position 287 of the JE envelope in ChimeriVaxTM-JE is permissive for the insertion of foreign DNA. We also provide evidence that amino acid positions 59, 231, 340, and 436 are permissive for insertion of foreign sequences.

Table 1 - PCR Primers

For cloning into pUC19:

TN1.F 5'-GCCGGTACCCACGATATCTCATGAAACTG-3'

TN2.R 5'-CTGCAGACCATCCCGAATTCTGGAAAATGG-3'

For mapping studies:

Not I/KAN-3 FP-2 5'-ACCTACAACAAAGCTCTCATCAACC-3'

Not I/KAN-3 RP-2 5'-TCCCGTTGAATATGGCTCATAAC-3'

TMOS.F 5'-CTGTCTCTTGTACACATCTTGCGGCCGC-3'

For cDNA synthesis using SUPERSCRIPT™ II RNase H Reverse Trascriptase (Life Technologies):

FNOR 5'-CCTGGGGAGAACACAAGGTTC-3'

YF 2.6- 5'-AAGAGGCTTTCACTATTGATG-3'

Table 2 - List of examples of pathogens from which antigens/peptides can be derived

VIRUSES:

Flaviviridae
- Yellow Fever virus
- Japanese Encephalitis virus
- Dengue virus, types 1, 2, 3 & 4
- West Nile Virus
- Tick Borne Encephalitis virus
- Hepatitis C virus (e.g., genotypes 1a, 1b, 2a, 2b, 2c, 3a, 4a, 4b, 4c, and 4d)

Papoviridae:
- Papillomavirus

Retroviridae
- Human Immunodeficiency virus, type I
- Human Immunodeficiency virus, type II
- Simian Immunodeficiency virus
- Human T lymphotropic virus, types I & II

Hepnaviridae
- Hepatitis B virus

Picornaviridae
- Hepatitis A virus
- Rhinovirus
- Poliovirus

Herpesviridae:
- Herpes simplex virus, type I
- Herpes simplex virus, type II
- Cytomegalovirus
- Epstein Barr virus
- Varicella-Zoster virus

Togaviridae
- Alphavirus
- Rubella virus

Paramyxoviridae:
- Respiratory syncytial virus
- Parainfluenza virus
- Measles virus
- Mumps virus

Orthomyxoviridae
- Influenza virus

Filoviridae
- Marburg virus
- Ebola virus

Rotoviridae:
- Rotavirus

Coronaviridae
- Coronavirus

Adenoviridae
- Adenovirus

Rhabdoviridae

Rabiesvirus

BACTERIA:

Enterotoxigenic *E. coli*
Enteropathogenic *E. coli*
Campylobacter jejuni

Helicobacter pylori

Salmonella typhi

Vibrio cholerae

Clostridium difficile
Clostridium tetani
Streptococccus pyogenes
Bordetella pertussis
Neisseria meningitides
Neisseria gonorrhoea
Legionella neumophilus

Clamydial spp.
Haemophilus spp.
Shigella spp.

PARASITES:

Plasmodium spp.
Schistosoma spp.
Trypanosoma spp.
Toxoplasma spp.
Cryptosporidia spp.
Pneumocystis spp.
Leishmania spp.

Table 3 - Examples of select antigens from listed viruses

VIRUS	ANTIGEN
Flaviviridae	
Yellow Fever virus	Nucleocapsid, M & E glycoproteins
Japanese Encephalitis virus	"
Dengue virus, types 1, 2, 3 & 4	"
West Nile Virus	"
Tick Borne Encephalitis virus	"
Hepatitis C virus	Nucleocapsid, E1 & E2 glycoproteins
Papoviridae:	
Papillomavirus	L1 & L2 capsid protein, E6 & E7 transforming protein (oncopgenes)
Retroviridae	
Human Immunodeficiency virus, type I	gag, pol, vif, tat, vpu, env, nef
Human Immunodeficiency virus, type II	"
Simian Immunodeficiency virus	"
Human T lymphotropic virus, types I & II	gag, pol, env

Table 3 - Examples of B and T cell epitopes from listed viruses/antigens

VIRUS	ANTIGEN	EPITOPE	LOCATION	SEQUENCE (5'-3')
			Flaviviridae	
Hepatitis C	Nucleocapsid	CTL	2-9	STNPKPQR
			35-44	YLLPRRGPRL
			41-49	GPRLGVRAT
			81-100	YPWPLYGNEGCGWAGWLLSP
			129-144	GFADLMGYIPLVGAPL
			132-140	DLMGYIPLV
			178-187	LLALLSCLTV
	E1 glycoprotein	CTL	231-250	REGNASRCWVAVTPTVATRD
	E2 glycoprotein	CTL	686-694	STGLIHLHQ
			725-734	LLADARVCSC
			489-496	CWHYPPRPCGI
			569-578	CVIGGVGNNT
			460-469	RRLTDFAQGW
			621-628	TINYTIFK
		B cell	384-410	ETHVTGGNAGRTTAGLVGLL TPGAKQN
			411-437	IQLINTNGSWHINSTALNCNESLNTGW
			441-460	LFYQHKFNSSGCPERLASCR
			511-546	PSPVVVGTTDRSGAPTYSWGANDTDV FVLNNTRPPL
		T helper	411-416	IQLINT
			Papoviridae	
HPV 16	E7	T helper	48-54	DRAHYNI
		CTL	49-57	RAHYNIVTF
		B cell	10-14	EYMLD
			38-41	IDGP
			44-48	QAEPD
HPV 18	E7	T helper	44-55	VNHQHLPARRA
			81-90	DDLRAFQQLF

What is claimed is:

What is claimed is:

1. A method for identifying a site in the envelope protein of a chimeric flavivirus or a genetically attenuated flavivirus that is permissive for insertion of a foreign peptide, said method comprising the steps of:

(i) introducing a nucleic acid molecule encoding a foreign peptide into a gene encoding a flavivirus envelope protein;

(ii) generating a flavivirus vector comprising an envelope protein encoded by said gene, wherein said envelope protein comprises said foreign peptide; and

(iii) determining whether the flavivirus vector generated in step (ii) is permissive for said insertion.

2. The method of claim 1, wherein said flavivirus vector is a chimeric flavivirus vector comprising the capsid and non-structural proteins of a first flavivirus and the pre-membrane and envelope proteins of a second flavivirus.

3. The method of claim 2, wherein said first flavivirus or said second flavivirus is selected from the group consisting of Japanese encephalitis, Dengue-1, Dengue-2, Dengue-3, Dengue-4, Yellow fever, Murray Valley encephalitis, St. Louis encephalitis, West Nile, Kunjin, Rocio encephalitis, Ilheus, ticke-borne encephalitis, Central European encephalitis, Siberian encephalitis, Russian Spring-Summer encephalitis, Kyasanur Forest Disease, Omsk Hemorrhagic fever, Louping ill, Powassan, Negishi, Absettarov, Hansalova, Apoi, and Hypr viruses, and said first and second flaviviruses are different flaviviruses.

4. The method of claim 1, wherein said foreign peptide comprises an epitope derived from an antigen of a viral, bacterial, or parasitic pathogen, or an epitope derived from a tumor-associated antigen.

5. The method of claim 1, wherein said nucleic acid molecule is introduced into said envelope gene randomly by transposon mutagenesis.

6. The method of claim 1, wherein determination of whether said flavivirus vector generated in step (ii) is permissive for said insertion is carried out by analysis of (a) the infectivity of said flavivirus vector, (b) the stability of the sequence of the foreign protein upon multiple passages of the vector, (c) the growth properties of said flavivirus vector, or (d) whether the flavivirus vector can be neutralized with antibodies against the envelope protein of said first flavivirus.

7. The method of claim 6, further comprising comparing the analysis of the flavivirus vector with a similar analysis of the flavivirus from which it was derived.

8. The method of claim 1, wherein said genetically attenuated flavivirus is Yellow Fever YF 17D.

9. A flavivirus vector comprising an envelope protein that comprises a foreign peptide.

10. The flavivirus vector of claim 9, wherein said vector is a chimeric flavivirus comprising the prM and E proteins of a first flavivirus and the C and non-structural proteins of a second flavivirus.

11. The flavivirus vector of claim 10, wherein said first flavivirus or said second flavivirus is selected from the group consisting of Japanese encephalitis, Dengue-1, Dengue-2, Dengue-3, Dengue-4, Yellow fever, Murray Valley encephalitis, St. Louis encephalitis, West Nile, Kunjin, Rocio encephalitis, Ilheus, ticke-borne encephalitis, Central European encephalitis, Siberian encephalitis, Russian Spring-Summer encephalitis, Kyasanur Forest Disease, Omsk Hemorrhagic fever, Louping ill, Powassan, Negishi, Absettarov, Hansalova, Apoi, and Hypr viruses.

12. The flavivirus vector of claim 9, wherein said foreign peptide comprises an epitope derived from an antigen of a viral, bacterial, or parasitic pathogen, or an epitope derived from a tumor-associated antigen.

13. The flavivirus vector of claim 9, wherein said vector comprises a genetically attenuated flavivirus.

14. The flavivirus vector of claim 13, wherein said genetically attenuated flavivirus is Yellow Fever YF 17D.

15. A pharmaceutical composition comprising the flavivirus vector of claim 9 and a pharmaceutically acceptable carrier or diluent.

16. Use of the pharmaceutical composition of claim 15 for the delivery of a peptide to a patient.

17. The use of claim 16, wherein the peptide is an antigen and said composition is administered to induce an immune response to a pathogen or tumor from which said antigen is derived.

18. A nucleic acid molecule comprising the genome of the flavivirus of claim 1 or the complement thereof.

Transposon mutagenesis in gene encoding the JE envelope

Fig. 1

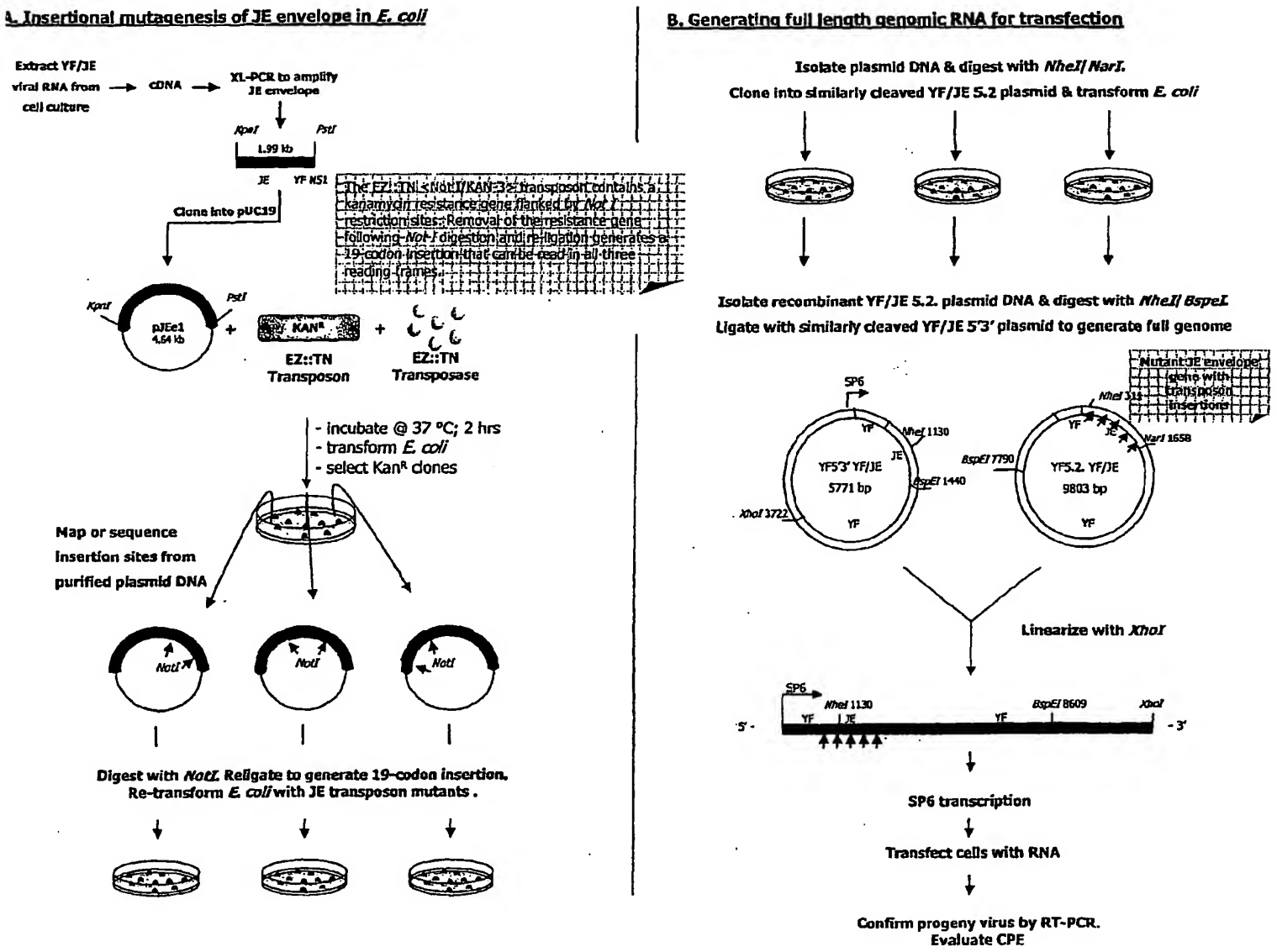


Fig. 2

1 2 3 4 5 6 7 8 9 10 11

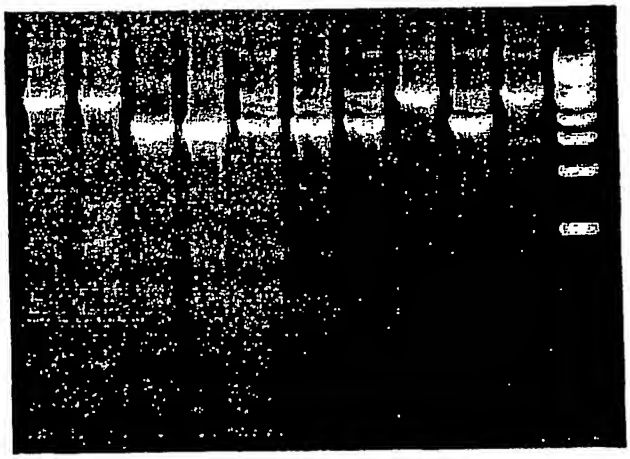


Fig. 3

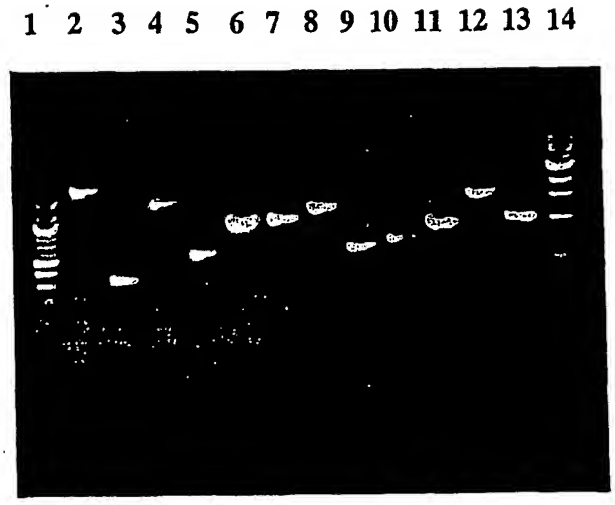


Fig. 4

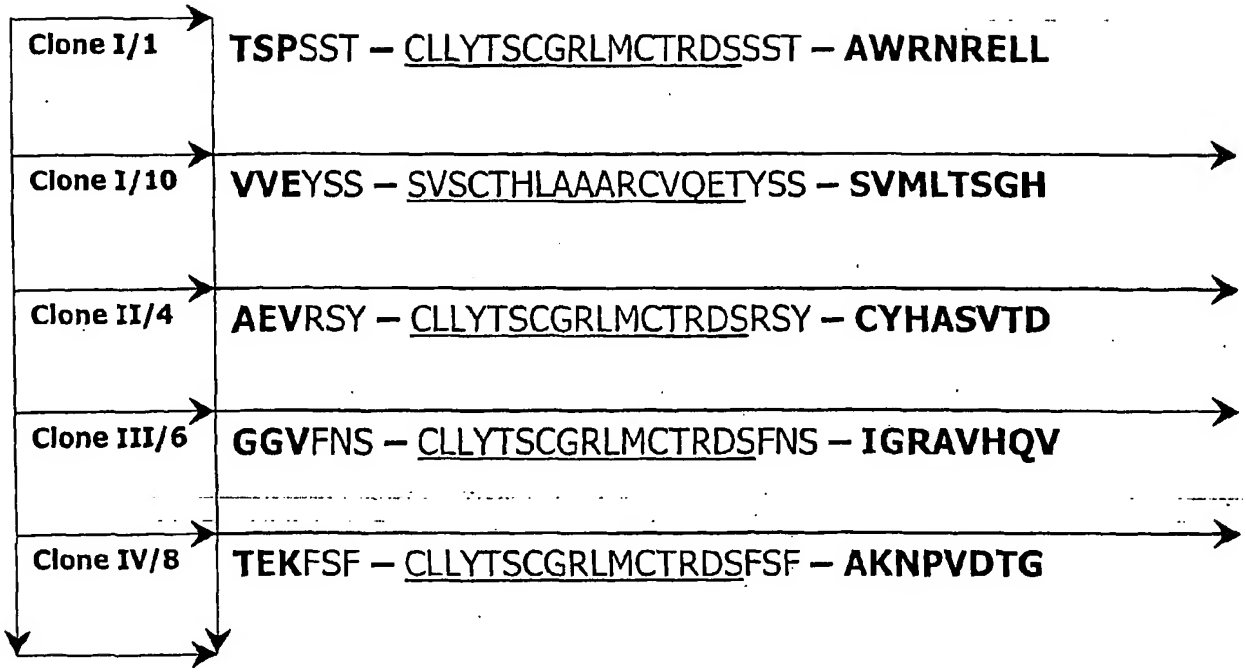

Clone I/1
TSPSST – CLLYTSCGRLMCTRDSSST – AWRNRELL
Clone I/10
VVEYSS – SVSCTHLAAARCVQETYSS – SVMLTSGH
Clone II/4
AEVRSY – CLLYTSCGRLMCTRDSRSY – CYHASVTD
Clone III/6
GGVFNS – CLLYTSCGRLMCTRDSFNS – IGRAVHQV
Clone IV/8
TEKFSF – CLLYTSCGRLMCTRDSFSF – AKNPVDTG

Fig. 5

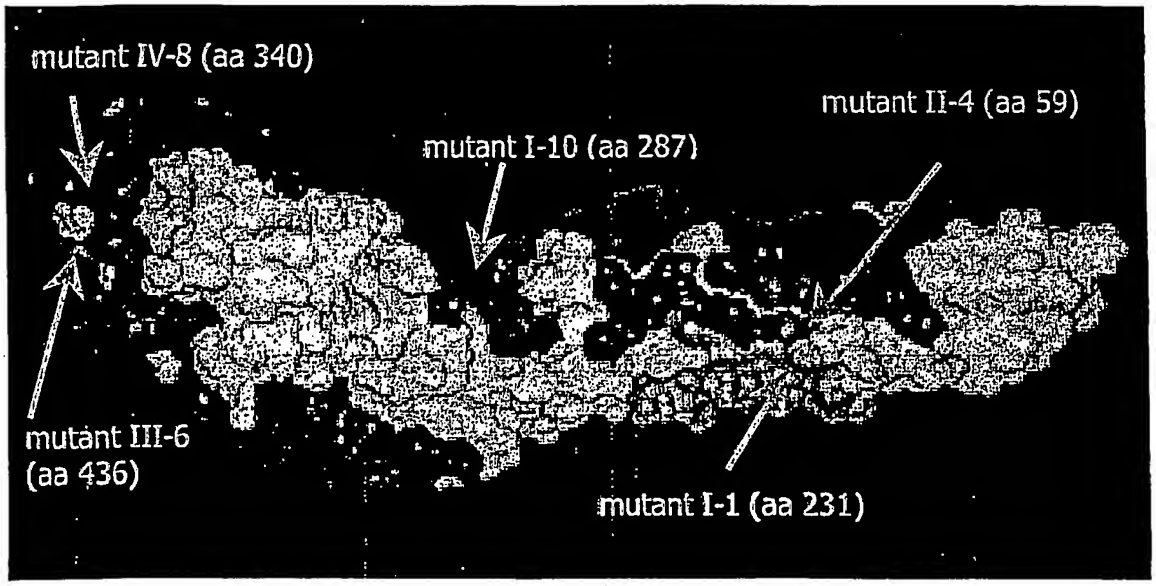

mutant IV-8 (aa 340)
mutant II-4 (aa 59)
mutant I-10 (aa 287)
mutant III-6
(aa 436)
mutant I-1 (aa 231)

Fig. 6

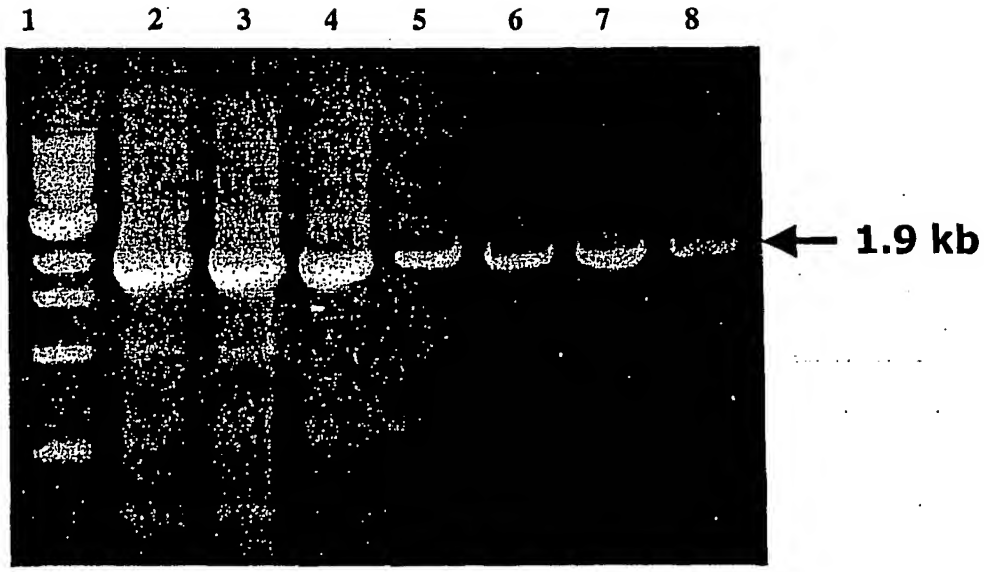


Fig. 7

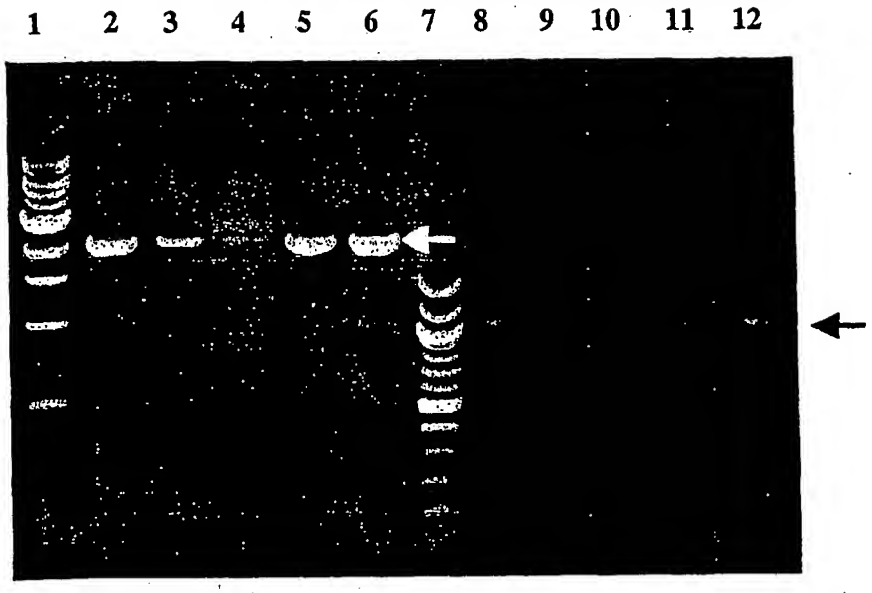


Fig. 8

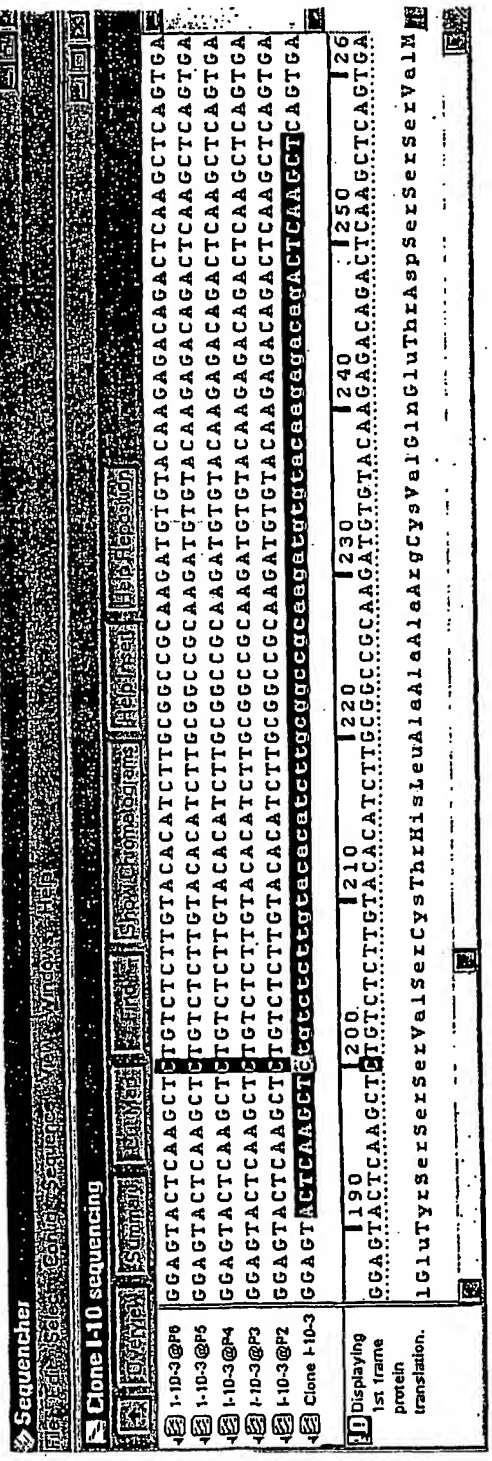

Sequencher
Clone I-10 sequencing
I-10-3@P6
I-10-3@P5
I-10-3@P4
I-10-3@P3
I-10-3@P2
Clone I-10-3
Displaying 1st frame protein translation.
190
200
210
220
230
240
250
260

Fig. 9

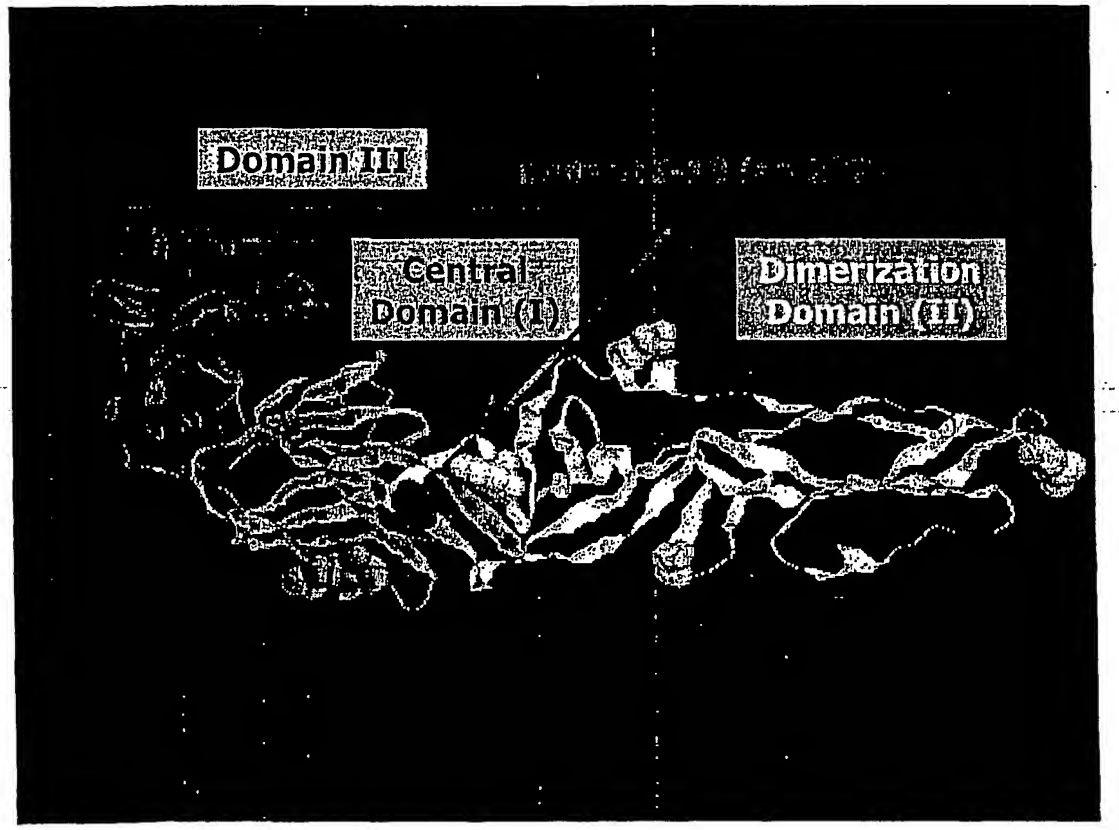


Fig. 10

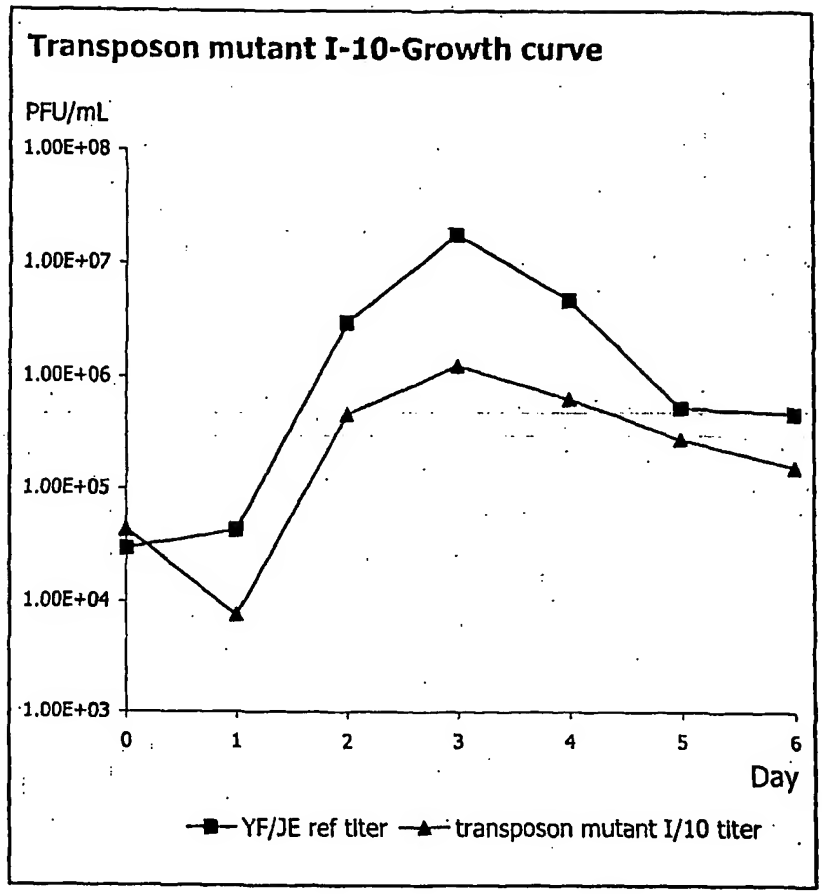

Transposon mutant I-10-Growth curve
PFU/mL
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
0
1
2
3
4
5
6
Day
YF/JE ref titer
transposon mutant I/10 titer

Fig. 11

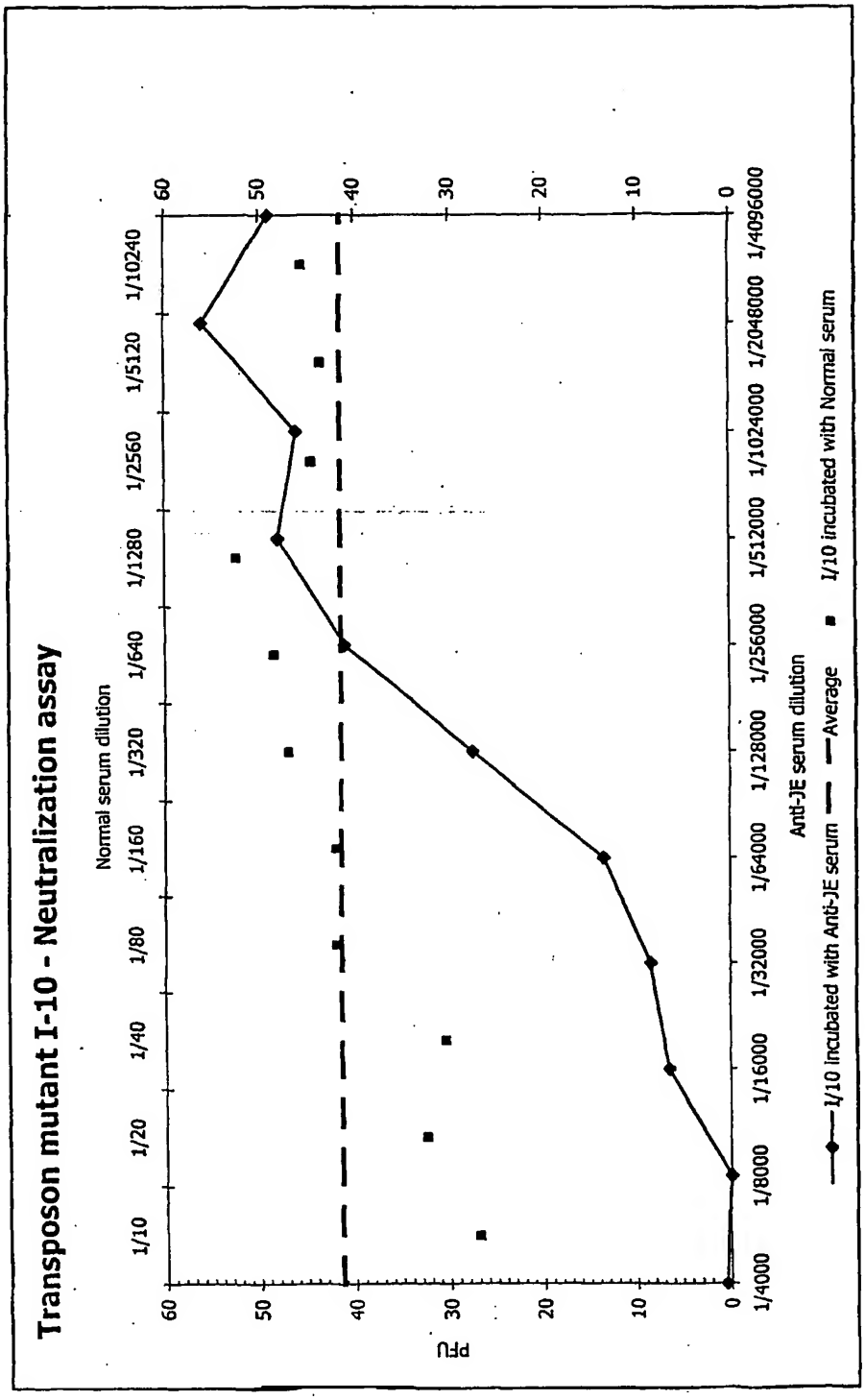
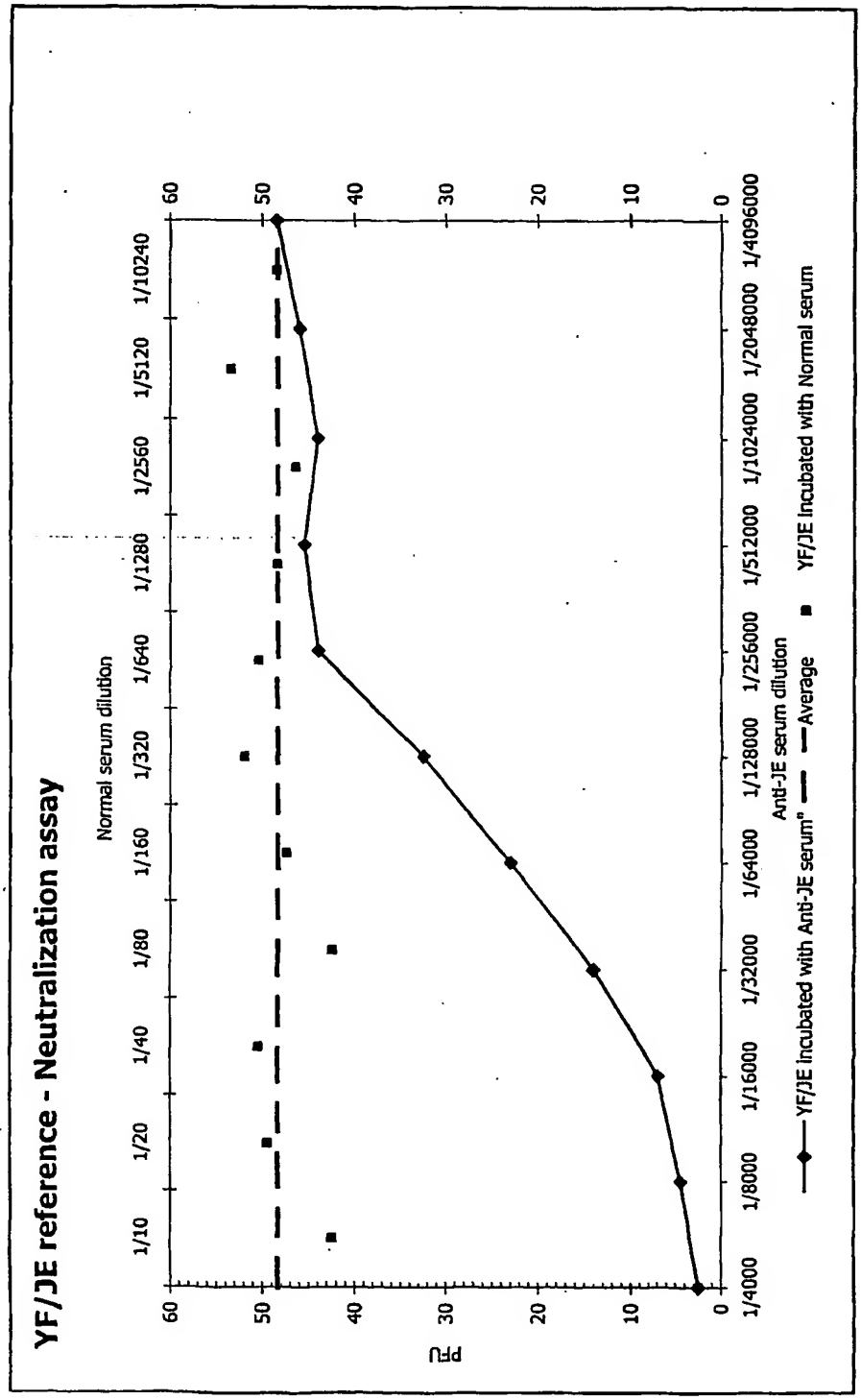

Transposon mutant I-10 - Neutralization assay
Normal serum dilution
1/10
1/20
1/40
1/80
1/160
1/320
1/640
1/1280
1/2560
1/5120
1/10240
PFU
60
50
40
30
20
10
0
1/4000
1/8000
1/16000
1/32000
1/64000
1/128000
1/256000
1/512000
1/1024000
1/2048000
1/4096000
Anti-JE serum dilution
1/10 incubated with Anti-JE serum
Average
1/10 incubated with Normal serum

Fig. 12


YF/JE reference - Neutralization assay
Normal serum dilution
1/10
1/20
1/40
1/80
1/160
1/320
1/640
1/1280
1/2560
1/5120
1/10240
PFU
60
50
40
30
20
10
0
1/4000
1/8000
1/16000
1/32000
1/64000
1/128000
1/256000
1/512000
1/1024000
1/2048000
1/4096000
Anti-JE serum dilution
YF/JE incubated with Anti-JE serum"
Average
YF/JE incubated with Normal serum